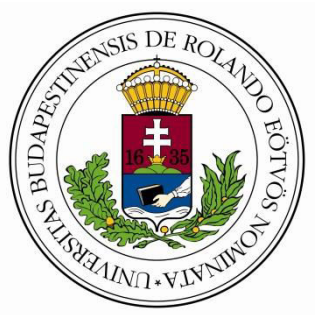
The characterization of the subcellular localization and function of the human ABCB6 protein

Doctoral Thesis (Ph. D.)

Katalin Kiss

Eötvös Loránd University, Faculty of Science
Doctorate School in Biology, Molecular Cell and Neurobiology Program



Supervisor: Dr. Gergely Szakács, Ph.D.
Leader of the Doctorate School: Prof. Dr. Anna Erdei
Leader of the Doctorate Program: Prof. Dr. Miklós Sass

Hungarian Academy of Science
Research Centre of Natural Sciences
Institute of Enzymology

Budapest
2014.

INTRODUCTION

ATP-binding cassette (ABC) transporters comprise a superfamily of membrane spanning multidomain proteins. ABC proteins are located in the membrane compartment of the cells, where they mediate translocation of various molecules across these barriers. Most human ABC proteins function as efflux pumps, translocating their substrates into the extracellular space or intracellular organelles. Human ABC transporters may be expressed in the plasma membranes of the cells, and also in intracellular membrane compartments including the endoplasmic reticulum (ER), lysosomes, peroxisomes and mitochondria. Based on sequence homology, 48 different ABC proteins (grouped into seven subfamilies ranging from A to G) have been defined in the human genome. The object of this thesis is the ABCB6 protein.

The first report describing ABCB6 function was based on the investigation of a *Saccharomyces cerevisiae* mutant strain lacking the mitochondrial ABC transporter Atm1p. Atm1p knockout cells accumulate high mitochondrial iron levels and are more sensitive to oxidative stress, iron-starvation and heavy metal toxicity. Based on the analysis of this complex phenotype, Atm1p is believed to transport a yet unknown substrate that has an important role in the biogenesis of cytosolic/nuclear iron-sulfur proteins as well as in regulating the overall mitochondrial iron homeostasis. In complementation studies using yeast strains lacking Atm1p, the human ABCB6 protein (formerly known as MTABC3) was shown to provide rescue from these phenotypic alterations. Based on this result, ABCB6 was suggested to be the human ortholog of Atm1p. In 2006, Krishnamurthy and colleagues reported that ABCB6 catalyzes the mitochondrial uptake of coproporphyrin III (CPIII) in mammalian cells, an oxidized derivative of the heme synthesis intermediate, thereby serving as an important regulator of cellular porphyrin biosynthesis.

For several reasons, we considered both suggestions controversial. In addition, the mitochondrial localization of ABCB6 was questioned in the literature, providing evidence for its localization in the endoplasmic reticulum, Golgi membranes, plasma membrane or vesicules, rather than in mitochondria. The observation of extra-mitochondrial localization has been extended by the study of dominantly inherited ABCB6 mutations in patients with ocular coloboma and ABCB6 was shown to carry the Lan (Langereis) blood group antigen system of the red blood cells' plasma membrane.

AIMS

- I planned to express the human ABCB6 protein and its non-functional mutant variant in heterologous insect (Sf9) cell system. In addition, I intended to perform functional characterization of ABCB6 by using various *in vitro* methods such as ATPase assay and azido-ATP occlusion/trapping methods.
- To elucidate the role of ABCB6 in cellular physiology, I aimed to analyze and establish the exact subcellular localization of the native and overexpressed ABCB6 protein in different cell lines.
- I also aimed to examine the role and function of the ABCB6 protein in erythroid differentiation. In addition, my aim was to screen the expression of ABCB6 in erythrocytes to find possible Lan- mutations in ABCB6 gene.
- Finally, I planned to examine the possible function and role of the unique N-terminal extension part of the human ABCB6 protein.

METHODS

Using a baculoviral expression system, we expressed wild type, non-functional (K629M) mutant variant and N-terminally truncated form (del205-ABCB6) of ABCB6 protein in Sf9 insect cells. Membranepreparation was applied and various *in vitro* methods were used as ATPase assay and azido-ATP assays (binding and "trapping").

K562 cells can be differentiated along the erythroid lineage by a variety of chemical compounds including hemin, and various antitumor agents. We investigated differentiating K562 cells treated with the targeted Bcr-Abl tyrosine kinase inhibitor, imanitib mesylate or hemin. De novo porphyrin biosynthesis can be readily followed experimentally by measuring the protoporphyrin IX (PPIX). Fluorescence of the cellsPPIX fluorescence was measured using an Attune cytometer, hemoglobin content of differentiating K562 cells was determined by the benzidine technique.

Wild type ABCB6 and its different variants were expressed in mammalian cells cells using lentiviral transduction or transient transfection. For the generation of ABCB6 knock-down cell lines, lentiviral particles were produced by following the manufacturer's instructions. To induce the expression of the shRNA constructs, IPTG (1 mM) was added to the cells.

instructions of the FuGENE HD Transfection kit (Promega).

To elucidate the subcellular localization of ABCB6 in the cells, different cell lines were used (HeLa, K562, HEK). Overexpressed and endogenous protein was analysed by differential centrifugation, co-immunoprecipitation, confocal microscopy and electronmicroscopy.

The constitutive active mutant of Rab5 (Q79L) was used to make endosomal structures visible by microscopy. The surface expression of ABCB6 protein on the cells was measured by OSK43 antibody.

The quantitative analysis of ABCB6 expression on red blood cell surface was made by antibody based FACS method. Experiements were performed by Dr. György Várady (MTA TTK), the sequencing of the ABCB6 soding region in samples showing lower ABCB6 protein expression was made Koszarska Magdalena (Hungarian Natianl Blood Transfusion Service). Nora Kucsma (MTA TTK) took part in generation of cell lines, dimerization assay and Western blot analysis.

RESULTS

- The wild type ABCB6 protein and the ABCB6(K629M) non-functional mutant variant both can be expressed in Sf9 cells, which suggest that these proteins are properly folded and integrated to the insect membrane.
- The human ABCB6 protein expressed in Sf9 cells can bind and hydrolise ATP, but has no measurable basal nor substrate-stimulated ATPase activity.
- The ABCB6(K629M) mutant can bind ATP but not hydrolize.
- ABCB6 is coregulated with the hemoglobin content of chemically induced K562 cells, but is dispensable for the erythroid differentiation of K562 cells.
- The overexpression or silencing of ABCB6 protein in chemically induced K562 cells does not influence the hemoglobin-content of the cells. This question the protein as the key regulator of erythroid differentiation and hem synthesis.
- Based on immunoaffinity and confoal studies ABCB6 is expressed in the endosomal/lysosomal compartment of the K562, HEK293T és HeLa cells and absent from mitochondria. We confirmed the mitochondrial localization of ABCB7, ABCB8 and ABCB10, suggesting that only these three ABC transporters should be classified as mitochondrial proteins
- Based on electronmicrocopy studies ABCB6 is located in the multivesicular bodies, multilamellar lysosomes and dens lysosomes.

- We confirmed the endo-lysosomal localization of ABCB6 in HeLa and K562 cells and showed that the protein is internalized from the plasma membrane through endocytosis, to be distributed to multivesicular bodies and lysosomes.
- ABCB6 is expressed as a full length glycoprotein in RBCs and released in exosomes from the reticulocytes during the final steps of erythroid maturation.
- ABCB6 is absent from the red blood cells' (RBC) surface of Lan- blood group.
- We examined the red blood cell (RBC) surface expression of ABCB6 by quantitative flow cytometry and confirmed high allele frequency of Lan-mutations. Our results suggest that genetic variants linked to lower or absent cell surface expression of ABCB6/Langereis may be more common than previously thought.
- Sequencing of the *ABCB6* gene in low RBC expressors identified a new allele (IVS9+1G>A, affecting a putative splice site at the boundary of exon 9) and two nonsynonymous SNPs, that are listed in the SNP database (R192Q (rs150221689) and G588S (rs145526996)).
- Based on HMMTOP topology prediction, ABCB6 contains a unique N-terminal transmembrane domain (N_0), which does not show sequence homology to known proteins.
- We found that the dimerization, membrane insertion and ATP binding/hydrolysis of the core ABCB6 complex devoid of N_0 is preserved.
- We found that N_0 has a crucial role in the endo-lysosomal targeting of ABCB6.

CONCLUSIONS

ABCB6 is known to be an outer mitochondrial protein which catalyzes the mitochondrial uptake of coproporphyrin III (CPIII) in mammalian cells, an oxidized derivative of the heme synthesis intermediate, thereby serving as an important regulator of cellular porphyrin biosynthesis. During my Ph.D. work I provided experimental evidence that indicate human ABCB6 is in fact an endo-lysosomal protein and absent from mitochondria. ABCB6 is coregulated with the hemoglobin content of chemically induced K562 cells, but is dispensable for the erythroid differentiation of K562 cells. In addition, the protein can be found on the surface of the cells, from where it is internalized via endosomes. Moreover, ABCB6 is present in the membranes of mature red blood cells and is released in association with exosomes during erythrocyte maturation. Molecular dissection of ABCB6 revealed that the N_0 and the

ABC core represent independently folding units, responsible for the lysosomal targeting and the ATPase activity of the full length protein, respectively.

Taken together, our results challenge the current view linking the expression and function of ABCB6 to mitochondria. Our results do not rule out the possibility that the ABCB6 protein has some yet unknown role in the erithroid maturation and/or the heme biosynthetic pathway but not responsible for mitochondrial porphyrin import. The endo-lysosomal localization of the protein questions the suggested function and also opens new opportunities to examine the real physiological function of the ABCB6 protein.

PUBLICATIONS

List of publications:

- **Kiss K**, Brozik A, Kucsma N, Toth A, Gera M, Berry L, Vallentin A, Vial H, Vidal M, Szakacs G: *Shifting the paradigm: the putative mitochondrial protein ABCB6 resides in the lysosomes of cells and in the plasma membrane of erythrocytes* PLOS One May 24, 2012
- Koszarska M, Kucsma N, **Kiss K**, Varady Gy, Gera M, Antalffy G, Andrikovics H, Tordai A, Studzian M, Strapagiel D, Pulaski L, Tani Y, Sarkadi B, Szakacs G: *Screening the expression of ABCB6 in erythrocytes reveals an unexpectedly high frequency of Lan mutations in healthy individuals* PLOS One, Accepted (September 2014)

Submitted publication:

- **Kiss K**, Kucsma N, Brozik A, Antalffy G, Tusnady G, Bergam P, van Niel G, Szakacs G: *Role of the N-terminal transmembrane domain in the endo-lysosomal targeting and function of the human ABCB6 protein*, Submitted to Journal of Biochemistry

Other publications:

- **Kiss K**, Katona M, Angyal V, Kucsma N, Sarkadi B, Takáts Z, Szakács G.: *A mass spectrometry based functional assay for the quantitative assessment of ABC transporter activity.* Rapid Commun Mass Spectrom. 2009 Nov;23(21):3372-6
- Kobolak J, **Kiss K**, Polgar Z, Mamo S, Rogel-Gaillard C, Tancos Z, Bock I, Baji AG, Tar K, Pirity MK, Dinnyes A.: *Promoter analysis of the rabbit POU5F1 gene and its expression in preimplantation stage embryos***.** BMC Mol Biol. 2009 Sep 4;10:88.